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ORIGINAL INVESTIGATION

Sunday Ene-ojo Atawodi · Kathrin Michelsen
Elmar Richter

Metabolism of a glucuronide conjugate of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in rats

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Abstract Besides 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-d-glucosiduronic acid (NNAL-Glu) is another important metabolite of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) which has been detected in the urine of tobacco users and non-smokers heavily exposed to sidestream cigarette smoke. In order to evaluate the toxicological significance of NNAL-Glu formation and excretion, the metabolism of [5-^{3}H]-NNAL-Glu was studied in rats. Five male F344 rats were administered 3.7 mg/kg [5-^{3}H]-NNAL-Glu by i.v. injection and the metabolites in urine analysed by HPLC. More than 90% of the radioactivity was excreted in urine within the first 24 h. Unchanged NNAL-Glu accounted for 81.2±3.1% of the total radioactivity; the remaining part of the dose appears to be deconjugated resulting in the urinary excretion of NNAL (3.6±1.7%) and its α-hydroxylation (11.5±2.2%) and *N*-oxidation (3.6±1.6%) products. The presence of α-hydroxylation products of NNAL-Glu in urine suggests that this NNK metabolite may be activated in vivo to carcinogenic intermediates.

Key words Tobacco-specific nitrosamine · 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone · [4-(Methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-d-glucosiduronic acid · Rat · Urine · Metabolism

Introduction

The tobacco-specific nitrosamine (TSNA), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been suggested to be involved in the induction of lung cancer in smokers (Hoffmann et al. 1993). In rodents, NNK is a potent lung carcinogen which also induces tumours of the nasal mucosa, exocrine pancreas and liver. NNK metabolism has been characterised in several animals as well as in human tissues and cells expressing human cytochrome P-450 isozymes showing that three major metabolic pathways exist (Fig. 1), namely carbonyl reduction, *N*-oxidation and α-hydroxylation (Crespi et al. 1993; Hecht et al. 1993a). Carbonyl reduction of NNK produces 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which induces tumours in the lung and exocrine pancreas (Hoffmann et al. 1993). NNAL is further metabolised to a glucuronide conjugate (NNAL-Glu). The pyridine nitrogen of both NNK and NNAL may be oxidised to the corresponding *N*-oxides which are devoid of carcinogenic activity. The pathway of primary importance for both NNK and NNAL carcinogenicity is hydroxylation of the methylene and methyl carbons adjacent to the *N*-nitroso group (so-called α-hydroxylation). In animal experiments the end products of α-hydroxylation, 4-oxo-(3-pyridyl)butyric acid (keto acid) and 4-hydroxy-(3-pyridyl)butyric acid (hydroxy acid), are the major urinary metabolites after administration of radiolabeled NNK (Morse et al. 1990; Hecht et al. 1993b; Richter and Tricker 1994).

NNAL and its glucuronide have recently been detected in the urine of smokers (Carmella et al. 1993; Hecht et al. 1994), tobacco chewers (Lazarus et al. 1994) and nonsmokers experimentally exposed to high concentrations of sidestream cigarette smoke (Hecht et al. 1993c). Since nothing is known about the biological significance of NNAL-Glu, an excretion study was performed by i.v. administration of NNAL-Glu to rats to determine whether this NNK metabolite is a genuine detoxification product or a putative active transport form of NNAL. The latter would have to be assumed if other NNAL- and/or NNK-derived metabolites are detected in either urine or faeces after administration of NNAL-Glu. Since NNAL-Glu is not synthetically available, it was isolated from the bile of rats treated with radiolabeled NNK.

S.E. Atawodi · K. Michelsen · E. Richter (✉)
Walther Straub-Institut für Pharmakologie und Toxikologie der Ludwig-Maximilians-Universität München, Nussbaumstrasse 26, D-80366 München, Germany

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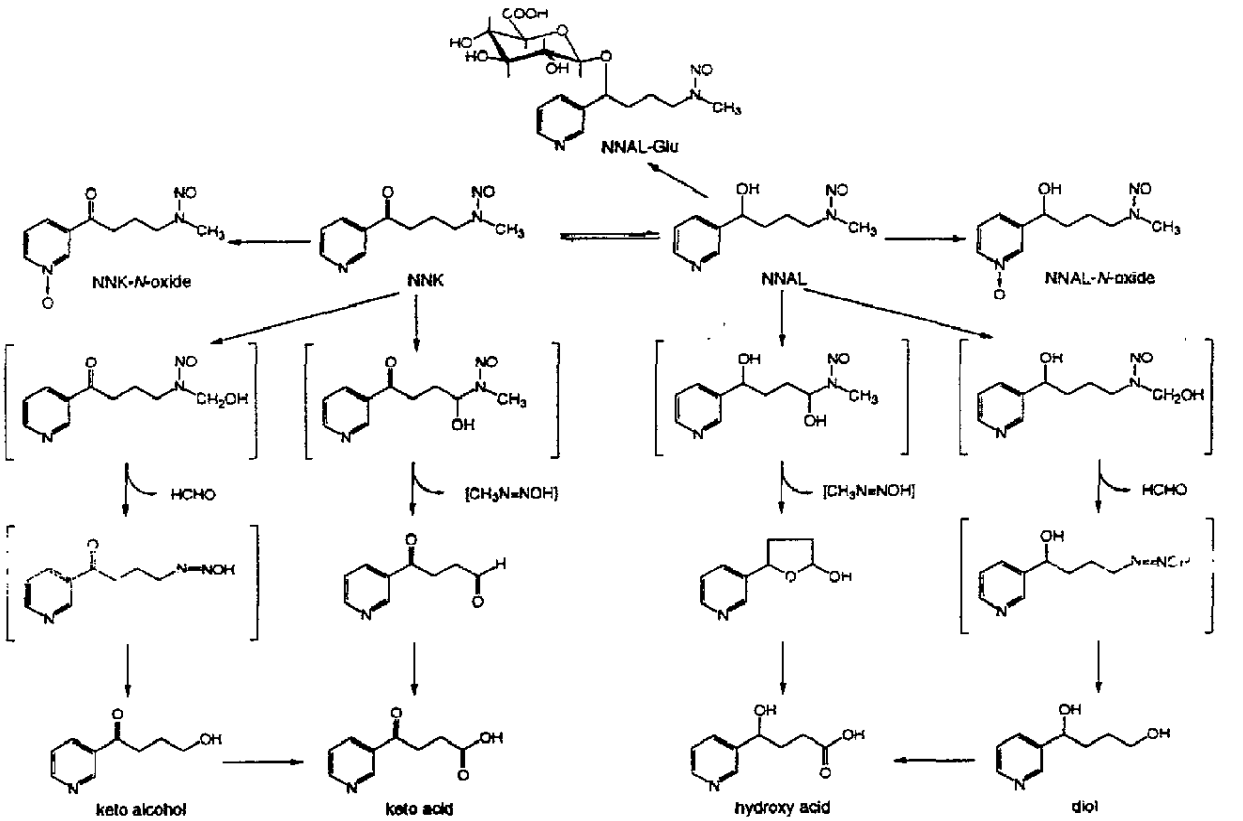

Fig. 1 Metabolic scheme of NNK. Structures in bracket are hypothetical intermediates (modified after Morse et al. 1990). *NNK* 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, *NNAL* 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, *NNAL-Glu* [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-d-glucosiduronic acid, *keto alcohol* 4-hydroxy-1-(3-pyridyl)-1-butanone, *diol* 4-hydroxy-1-(3-pyridyl)-1-butanol, *keto acid* 4-oxo-(3-pyridyl)butyric acid, *hydroxy acid* 4-hydroxy-(3-pyridyl)butyric acid

Materials and methods

Chemicals

[5-^{3}H]NNK with a specific activity of 2.89 Ci/mmol and radiochemical purity of >99% as well as unlabeled NNK were obtained from Campro Scientific (Emmerich, Germany). NNK metabolite standards were a gift from D. Hoffmann (American Health Foundation, Valhalla, N.Y.). All other chemicals were of either HPLC or analytical grade and were purchased from Merck (Darmstadt, Germany).

Animals

Male Fischer F344 rats with a body weight of about 200 g were obtained from Charles River (Sulzfeld, Germany). They were allowed free access to Alma h 1003 Laboratory Chow (F.Botzenhardt KG, Kempten, Germany) and drinking water, and were maintained in groups of five in metal cages under standard laboratory conditions (20±2° C; 50±10% relative humidity; 12-h light/dark cycle). The animal experiments were officially approved by the Government of Upper Bavaria (AZ 211-2531-53/92).

Isolation of NNAL-Glu from rat bile

Bile was collected from anaesthetised rats as described previously (Schulze et al. 1992). [5-^{3}H]NNK diluted by unlabeled NNK was dissolved in saline for intraperitoneal administration (240 µmol/kg, 0.336 Ci/mol). The bile was collected for 8 h. After dilution of the bile by 1 vol phosphate buffer (20 mM, pH 7.2), aliquots of 250–500 µl were analysed by HPLC as described below and the fraction containing NNAL-Glu collected. The combined fractions were freeze dried, taken up in phosphate buffer and fractionated again by HPLC. NNAL-Glu was collected, freeze dried and dissolved in phosphate buffer to give a final concentration of 6 µmol/ml (4.5 × 10^6 dpm/ml). The radiochemical purity of NNAL-Glu was >99.5% as determined by HPLC. The identity of NNAL-Glu was confirmed by cochromatography with [1-^{14}C]NNAL-Glu which was characterized by thermospray LC-MS and enzymatic treatment (Schulze et al. 1992).

Metabolism of NNAL-Glu

NNAL-Glu was administered i.v. via the tail vein to five rats. The exact dose was determined gravimetrically by weighing the syringe before and after injection. On average 3.7 mg/kg (9.6 µmol/kg) was administered (range 5–15 µmol/kg body weight). The animals were placed in stainless steel metabolism cages and urine and faeces collected separately. The urine was collected on ice. The first sampling was after 6 (four of five rats), 24, 48 and 72 h. Total radioactivity in urine was determined by liquid scintillation counting (Packard 2500 TR, Canberra, Frankfurt, Germany) with external standardisation. Faeces was collected daily, homogenised with 10–20 ml methanol and the

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total radioactivity determined in 500 µl aliquots of the methanol by liquid scintillation counting. Urine samples from the first two sampling periods (0–6 and 6–24 h) were analysed by HPLC for NNAL-Glu and metabolites after addition of 1/10 the volume of phosphate buffer (200 mM, pH 7.2).

HPLC analysis

Bile and urine samples were chromatographed on a 4.6 × 250 mm LiChrosorb RP18 SelectB column (Merck, Darmstadt, Germany) using a gradient of 100% A for 0.5 min, linear to 80% A/20% B in 20 min, linear to 20% A/80% B in 2 min and linear to 10% A/90% B in 0.5 min (A: 20 mM phosphate buffer, pH 7.2; B: acetonitrile) at a flow rate of 0.7 ml/min. ^{3}H was detected by solid phase radioactivity monitoring (Ramona, Raytest, Straubenhardt, Germany). Radioactive metabolites were identified by cochromatography with unlabeled reference compounds using UV detection at 254 nm.

Results and discussion

[5-^{3}H]NNAL-Glu was isolated from the bile of eight rats administered 240 µmol/kg [5-^{3}H]NNK. Within 7 h the rats excreted about 20–30% of the radioactivity in the bile. NNAL-Glu made up >90% of the radioactiviy in bile, confirming the results of our previous study on the biliary excretion of NNK (Schulze et al. 1992). After purification by HPLC the chromatographic purity of NNAL-Glu was >99.5%.

Another group of five rats was treated by i.v. administration of 1.8–5.7 mg/kg [5-^{3}H]NNAL-Glu. The results for the urinary excretion of NNAL-Glu and its metabolites are presented as the mean ± SE of all five rats with the exception of the first two sampling intervals (0–6 h and 6–24 h), since urine from one rat was only obtained after 24 h (Table 1). Within 3 days after i.v. administration of [5-^{3}H]NNAL-Glu, 98.9 ± 1.4% of the total dose of ^{3}H was recovered in urine and faeces. The bulk of ^{3}H was excreted in urine during the first day (93.9 ± 0.94%) and more than half of this amount within the first 6 h (Table 1). Only minor amounts of ^{3}H were excreted in urine on the second and third day (2.30% and 0.61%) and on the first and second day in faeces (1.35% and 0.73%). The small amounts of ^{3}H excreted in urine on days 2 and 3 and in faeces did not allow the determination of NNAL-Glu and metabolites by HPLC.

The metabolic profile in urine changed significantly over the first 24 h. NNAL-Glu accounted for 93% of total ^{3}H in urine within the first 6 h and decreased to 53% in the second sampling period between 6 and 24 h (Table 1). Over the same period hydroxy acid, the major metabolite of NNAL-Glu and end product of NNAL α-hydroxylation (Hecht et al. 1993a), increased sevenfold from 3.9% to 26.7%. The excretion of both NNAL and its detoxification product, NNAL-*N*-oxide, were increased eightfold in the second sampling period. This suggests that NNAL-Glu is taken up from the bloodstream into the liver, excreted with bile, and cleaved by intestinal glucuronidase to NNAL which is reabsorbed and undergoes further metabolism (Adams et al. 1985; Hecht and Trushin 1988). This would explain the presence of small amounts of NNK (Adams et al. 1985) and its α-hydroxylation product, keto acid, in the urine of rats treated with NNAL-Glu.

Table 1 Metabolic profile in urine following a single i.v. injection of 3.7 mg/kg NNAL-Glu

Metabolite	Percent of the dose excreted in urine after[a]		
	0–6 h	6–24 h	0–24 h
Hydroxy acid	2.3 ± 1.1 (3.9)	8.0 ± 0.5 (26.7)	9.9 ± 0.7
Keto acid	0.4 ± 0.3 (0.8)	0.6 ± 0.2 (1.9)	0.9 ± 0.4
NNAL-Glu	54.5 ± 7.0 (93.0)	21.5 ± 8.5 (53.0)	76.1 ± 1.4
NNAL-*N*-Oxide	0.6 ± 0.2 (1.0)	2.2 ± 0.4 (7.9)	3.3 ± 0.7
NNAL	0.9 ± 0.5 (1.3)	2.9 ± 0.3 (9.9)	3.3 ± 0.7
NNK	0.1 ± 0.1 (0.1)	0.2 ± 0.1 (0.7)	0.2 ± 0.1
Sum	58.8 ± 7.6	35.2 ± 8.2	93.9 ± 0.9

[a] Mean ± SE of total administered dose in four (0–6 h and 6–24 h) and five (0–24 h) rats, respectively. Percentage of total ^{3}H in the sample is given in parentheses

NNAL and NNK are equipotent lung carcinogens in the F344 rat, in which NNAL induces fewer tumours of the liver and nasal mucosa but more tumours of the exocrine pancreas compared to NNK (Rivenson et al. 1988). For both NNK and NNAL, α-hydroxylation has been shown to be a key step in the induction of tumours (Hecht et al. 1993a). The presence of α-hydroxylation products (keto acid and hydroxy acid) in the urine of rats after i.v. administration of NNAL-Glu indicates that this NNK metabolite can be activated to a carcinogen when present in blood.

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